NONHOST RESISTANCE IN *Arabidopsis thaliana* (*col-0,ler-0,npr,pen2 gfp*) AGAINST *Magnaporthae oryzae"*

Thesis submitted to Department of life science for the partial fulfillment of the M.Sc. Degree in Life science

Submitted By
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DECLARATION

I do here by declare the project report entiteled "**NONHOST RESISTANCE IN *Arabidopsis thaliana* (*col-0,ler-0,npr,pen2 gfp*) AGAINST *Magnaporthae oryzae*"** submitted to the life science dspartment,National institute of technology,Rourkela for the partial fulfilment of master degree in life science is a faithfull record of bonafide and original record work carried out by me under the guidance and supervision of Dr.Binod Bihari Sahoo ,assistance professor ,department of life science,NIT,Rourkela.

Dt:10-05-2015
NIT,Rourkela

Abhipsa Bhoi

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CERTIFICATE

This is certified that the thesis entitled "N**onhost resistance in *Arabidopsis thaliana* (*Col-0, Ler-0 and npr*) against *Magnaporthae oryzae*"** which is being submitted by Ms. Abhipsa Bhoi, Roll No-413LS2025, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other institution and university for the award of any degree or diploma.

09/05/2015

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ABHIPSA BHOI

CONTENTS

LIST OF ABBREVIATION

PRRs.....pattern recognition receptors

MAMPS..... microbial/pathogen associated molecular pattern

NB.....-nucleotide binding

LRR.....leucine rich repeat

PTI.....pathogen triggered immunity

ETS.....effector triggered susceptibility

ETI.....effector triggered immunity

HR.....hiper sensitive

M. oryzae.....Magnaporthe oryzae

Col-0.....colombia

NHR.....nonhost resistant

AVR..... avirulence

PDA.....patato dextrose agar

LER .....landsberg erecta

ABSTRACT

Nonhost resistance is an expansive range plant protection that gives safety to all individuals from a plant animal types against all detaches of a microorganism that is pathogenic to other plant species. After arriving on the surface of a nonhost plant animal categories, a potential fungal pathogen at first experiences preformed and, later, activate plant safeguards. One of the introductory guard reactions from the plant is pathogen-related sub-atomic example (PAMP)-activated resistance (PTI). Nonhost plants likewise have components to distinguish nonhost-pathogen effectors and can trigger a barrier reaction alluded to as effector-activated invulnerability (ETI). This nonhost resistance reaction frequently brings about an excessively touchy reaction (HR) at the contamination site. This review gives a diagram of these plant safeguard methodologies. We count plant qualities that present nonhost resistance and the fungal counter-protection methods. Furthermore, prospects for utilization of nonhost imperviousness to accomplish expansive range and sturdy resistance in harvest plants .

INTRODUCTION:

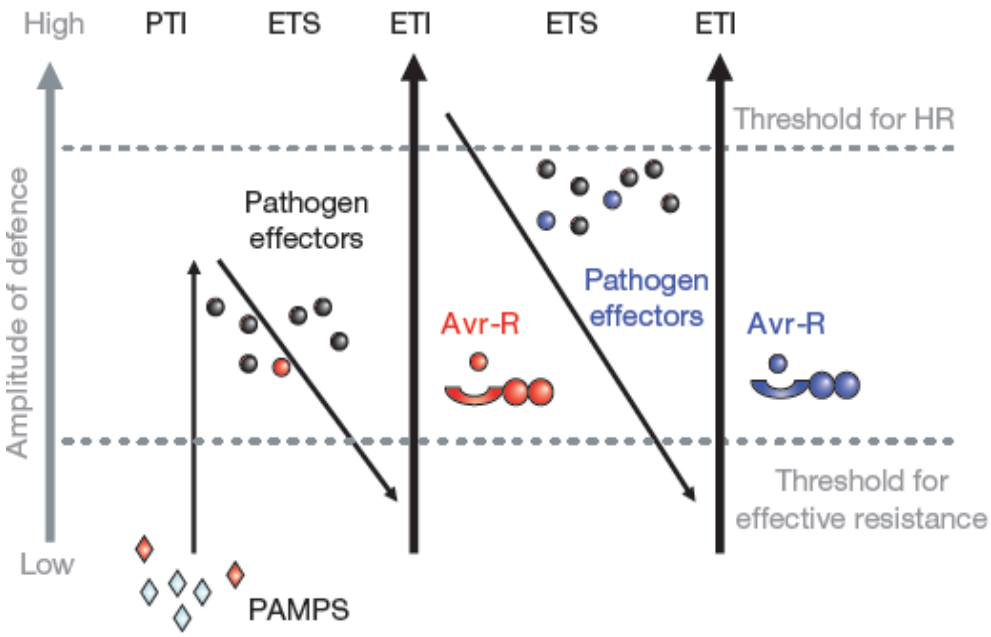
In nature there are various types of pathogen which infect many plants. According to the interaction between pathogen and plant; plants are act as a host or non host. The plants which are easily infected by some pathogen they are called host and some of the plants which show resistance against the pathogen they are called nonhost. So there are many plants which having their own defence system and fight against the pathogen to show their nonhost resistance. It is expressed by every plant towards the majority of potentially pathogenic microbes. Nonhost resistance is the most common form of disease resistance exhibited by plants. Nonhost resistance to fungi, at least, generally seems be under complex genetic control and can involve a mutiplicity of defense factors that, individually, may segregate within the species without compromising overall resistance. Such resistance contrast with host resistance, which is expressed by plant genotypes within an otherwise susceptible host species. Host resistance is usually parasite-specific in that, it is restricted to a particular pathogen species and commonly is expressed against specific pathogen genotypes. Variation in host resistance is often controlled by the segregation of single *resistance* (*R*) genes, the products of which directly or indirectly interact with 'specific elicitors' produced by the pathogen and coded for by *avirulence* (*avr*) genes .

plant contain innate immunity of each cell and on systemic signal which protect from infection site. We previously know disease resistance(R)protein diversity, polymorphism at R loci in wild plants and lack thereof in crops and the suit of the cellular responses that follow R protien activation. So here we concluded that the many of the R protiens might be activated by pathogen encoded effectors but not by the direct recognition. So this 'guard hypothesis' says that R protiens indirectly recognise pathogen effectors by monitoring the integrity of host cellular target of the effectors action. The concept is that R protien is recognise the pathogen induced modified itself is similar to the recognition of modified itself in 'danger signal' model of mammalian immune system.

It is presently pass that there are, generally, two branches of the plant insusceptible framework. One uses transmembrane pattern recognition receptors (PRRs) that react to microbial- or pathogen-related atomic examples (MAMPS or PAMPs, for example, flagellin6. The second demonstrations to a great extent inside the cell, utilizing the polymorphic NB-LRR protein items encoded by most R genes1. They are named after their

trademark nucleotide tying (NB) and leucine rich rehash (LRR) spaces. NB-LRR proteins are extensively identified with creature CATERPILLER/NOD/NLR proteins7 and STAND ATPases8. Pathogen effectors from different kingdoms are perceived by NB-LRR proteins, and enact comparative resistance reactions. NBLRR-interceded ailment resistance is compelling against pathogens that can become just on living host tissue (commit biotrophs), or hemibiotrophic pathogens, yet not against pathogens that kill the host tissue during colonization.

Our current perspective of the plant safe we can be shown to as a four phased 'zigzag' model (Fig.1),in which we introduce several vital shortened forms. In stage 1, PAMPs (or MAMPs) are recognized by PRRs, which results in PAMP-triggered immunity (PTI)thatcan end further colonization. In stage 2, then the pathogens convey effectors that add to pathogen destructiveness. Effectors can intrfere with PTI. This outcomes in effector-triggered susceptible (ETS). In stage 3, a given effector is 'particularly perceived' by one of the NB-LRR proteins, bringing about effector-activated insusceptibility (ETI). Recognition is either in direct,or throughdirectNB-LRRrecognition of an effector. ETI is a quickened and opened up PTI reaction, bringing about disease resistance and, ordinarily, an extremely touchy cell passing reaction (HR) at the disease site. In stage 4, regular determination drives pathogens to stay away from ETI either by shedding or expanding the perceived effector quality, or by procuring extra effectors that smother ETI. Characteristic choice results in new R specificities so that ETI can be activated once more. Beneath, we survey every stage thusly, we consider future difficulties in understanding and controlling the plant immune system.



(**FIGURE:1 non host resistance mechanism)**

After all the two types of plants are there i.e (1)wild type plants and (2)mutant type plants.

Wild type-genotype or pnenotype which is found in the nature or in the laborotary stock for a given organism. Wild type plants are cannot be infected by pathogen.so it shows its resistance mechanism to plants.

Mutant type-mutation is the process of breeding which exposing its seeds to a chemical or a radiation to generate a mutants with a desirable trait to be bred with other cultivar. in case of mutant plants it easily infected by the pathogen so it is pathogen susceptible.

For example: Colombia ,ler,NPR,pen2-gfp plants are under go wild type plants which are cannot be infected by pathogen.

Review of literature:

Why Arabidopsis thaliana taken as a model

Arabidopsis thaliana was the first plant, and the third multicellular life form after Caenorhabditis elegans (The C. elegans Sequencing Consortium 1998) and Drosophila melanogaster (Adams et al. 2000), to be totally sequenced (The Arabidopsis Genome Initiative 2000). At the time, it was asserted that the Arabidopsis genome succession "...

makes the potential for immediate and productive access to a much more profound comprehension of plant improvement and ecological reactions, and grants the structure and progress of plant genomes to be surveyed and caught on."

Arabidopsis thaliana is a little blossoming plant that is broadly utilized as a model organic entity as a part of plant science. Arabidopsis is an individual from the mustard (Brassicaceae) family, which incorporates developed species, for example, cabbage and radish. Arabidopsis is not of major agronomic criticalness, but rather it offers imperative focal points for fundamental research in hereditary qualities and sub-atomic science.

Some useful statistics:

We use Arabidopsis as a model because of the several reason:

- It has the smallest ginome in plant kingdom.

- The life cycle is too short –about 6 weeks from germination to seed maturation.
  It followed efiicient transformation method using Agrobacterium tumefaciens.
  Seed production is prolific and the plant is easily cultivated in restricted space.
- Transformation is successful by utilizing *Agrobacterium tumefaciens*5) Mutations can be easily generated (e.g., by irradiating the seeds or treating them with mutagenic chemicals)
- It isnormally self-pollinated so recessive mutations quickly become homozygous and thus expressed.
  This behabioral properties of Arabidopsis thaliana was studied by Multinational research community academic.

So all this properties or advantages maid the Arabidopsis as a model for our experiment.

History of Arabidopsis thaliana as a research organism:

Arabidopsis first discovered by Johansen Thal in the Harz mountains in the sixteenth century so that he called Pillosella siliquosa. Oin early stage of a mutant was in 1873 ,F Liabach first describe the potential of Arabidopsis thaliana as a model organism for genetics in 1943.he did

some work on this much earliar so that he publish its correct chromosome in 1907.after that the first collection of large indused mutant was mad by Libach's student E .Reinholz and her thesis was submitted on 1945 and it is published on 1947.Landridge plated an important role in establishing the properties o& utility ogf the organism for the laborotary studies same as the Redie and others did (such as J.H. van der Veen in the Netherlands, J. Veleminsky in Czechoslovakia and G. Röbbelen in Germany) in 1960s. Here the important thing is that Radie was write a review on Arabidopsis thaliana. So we go through bibliographica genetica vol 20,no:2, 1997.after that one more review of Radie is Ann. Rev. Genet. (1975) vol. 9,111-127. So here both of this review paper are go through the use of Arabidopsis in laborotary.

(FIGURE:2 *ARABIDOPSIS THALIANA)*

Time line growth for Arabidopsis thaliana:

Time table of development stages decided for Arabidopsis ecotype Columbia-O from Boyes, et.Hal. (2001) The Plant Cell 1499-1510.

View a period slip motion picture of a developing Arabidopsis seed, from 0 to 65 hours in the wake of planting. Ecotype is Col-0, pictures were caught like clockwork. This Quicktime film was charitably given to TAIR by Dr. Ronny Joosen (Wageningen University).

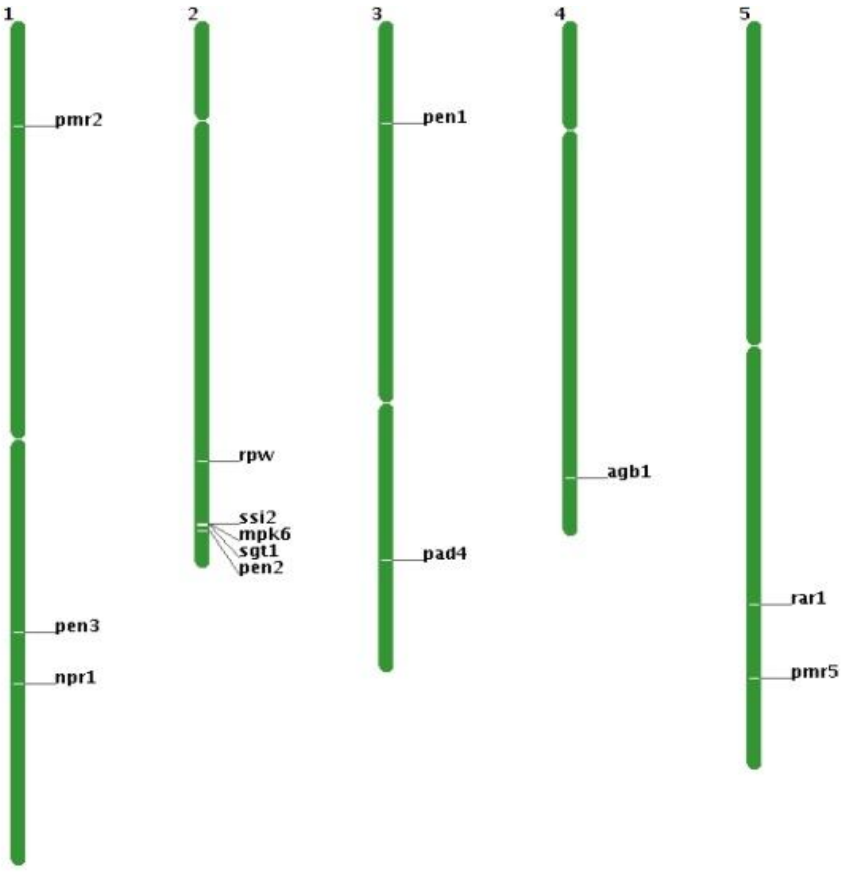
View a period slip motion picture demonstrating the development of Arabidopsis from 4 days in the wake of planting (dap) to 22 dap. Columbia plants were developed in steady light in a development chamber; pictures were caught at regular intervals. This 65MB Quicktime feature compasses occasions taking after germination up til blasting. The first feature was thoughtfully given to TAIR by Dr. Scratch Kaplinsky (Swarthmore College, PA) .**Classification of Arabidopsis thaliana:**

(**FIGURE:3 *Arabidosis Thaliana* in growth chamber)**

Kingdom-	Plantae (plant)
Subkingdom-	Tracheobionta (vascular plant)
Superdivision-	Spermatophyta (Seed plants)
Division-	Magnoliophyta (flowering plants)
Class-	Magnaliopsida (Dicotyledons)
Subclass-	Dilleniidae
Order-	Capparales
Family-	Brassicaceae (mustard family)

Genus- Arabidopsis Heynh (rock cress) Species-Arabidopsis thaliana (L.) Heynh. (mouseear cress)

Arabidopsis has different types of gene in there five chromosome such as pen1,pen2,pen3,NPR, MPK6,pmr2 etc.so the gene on the chromosomes structure are shown below:



(FIGURE: 4- Chromosome map of *Arabidopsis thaliana* NHR genes.)

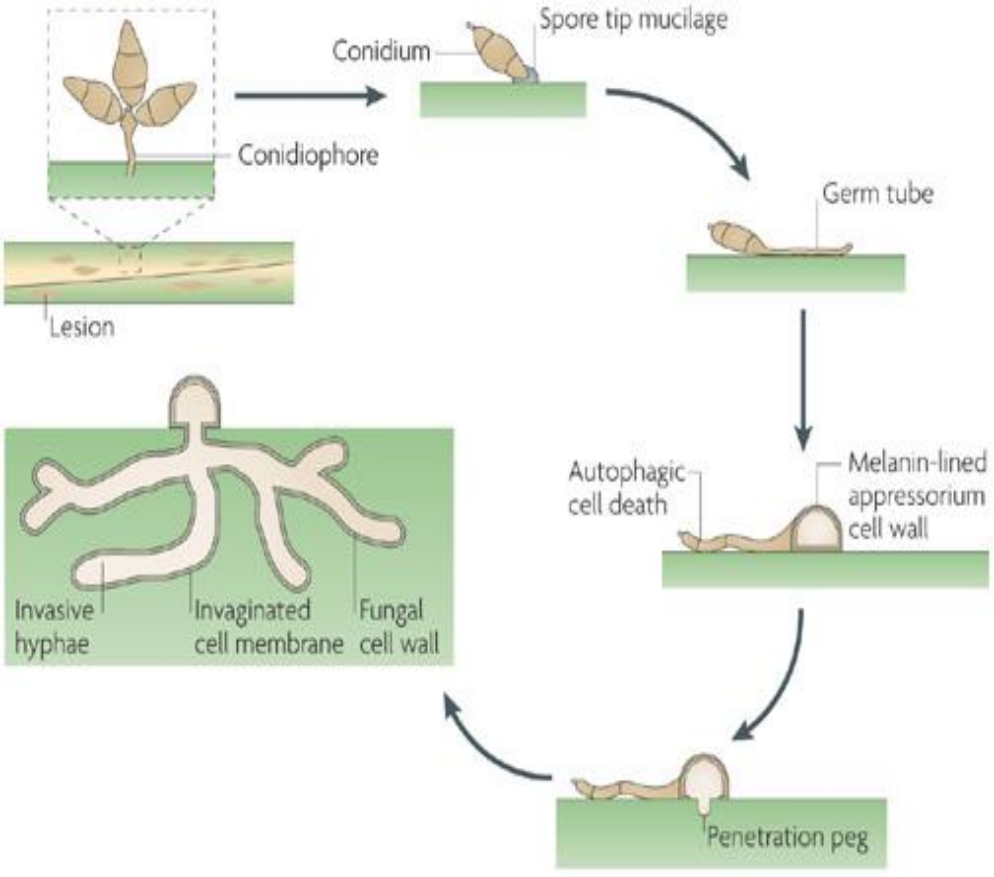
Magnaporthe oryzae:

Magnaporthe oryzae (anamorph: Pyricularia grisea) otherwise called rice impact organism.which is an essential plant pathogen separated from rice and an assortment of other rice field weeds. It influences all development phases of the plant with extreme harm amid the seedling stage. This growths creates spores that can undoubtedly be scattered by wind and sprinkling precipitation. The spores can spend the Winter in rice grains and rice stubble and can contaminate new products the accompanying year. Contamination is more probable over long stretches of downpour or high dampness. There are known strains of rice impervious to this sickness that may be useful for it control. Magnaporthe oryzae is the most imperative rice pathogen overall known to happen in 85 nations. Consistently, the misfortunes in products

because of rice impact could nourish 60 million individuals. The Magnaporthe oryzae genome was discharge as a major aspect of the Magnaporthe similar database, it as size of 41.03 Mb and encodes around 12,593 protein-coding qualities.

The rice–M. oryzaepathosystem has consider as a best model for plant–microbe interactions study.M.oryzainfect the rice by following many developmental process. In the first developmental process, a germ tube produed from conidium and grow towards a infectious structure called appressorium.Appressorium secrete a substance like mucilage by which it tightly adheres to the surface of the plant. Fungus produces turgor pressure under the appressorium which is melanin lined ,due to this turgor pressure a narrow penetration peg is produced towards the host surface. Fungus able to enter into a leaf epidermal cell through penetration peg. After entry, the peg give rise to bulbous and lobed infectious hyphae which grow intra- and intercellular.

Life cycle of M.oryza:



(FIGURE: 5- Life cycle of *M. Oryzae)*

The rice blast or the Magnaporthe oryzae fungus starts its infection cycle when a three – called conidium lands present on the leaf surface of the plant.the spores ara attaches to the hydrophobic cuticle and starts germinating to produce a narrow germ tube,which subsequently flattens and hooks at the tip of the surface before differentiating it into appressorium so the single called appressorium matures and the three conidium collapses and it going to dies in a proogammed process which requires autophagy.after that the appressorium becomes melanized and it developes substantial turgor.this turgor translated into physical force and forming a narrow penetration peg at the base,and allow the entry into the rice epidermis.plant tissue invation occurs by means of the bulbous and invasive hyphae which can invaginate the rice plasma membrane and invade the epidermal cells.so here cell to cell movement can initially occur by plasmodesmata.so so the disease is occure between 72 and 96 hours after infection and sporulation occures under humid condition.

Example of Plant defence mechanism:

Prieviously we tought about NB-LRR protein so here breifely explain about it :

NB-LRR protein and pathogen interaction are two types:

1)direct interaction 2)indirect interaction.

The first evidence for direct interaction studies proved by studied of Pi-ta and R gene from rice that show resistance to specific strain of the rice blast fungus *Magnaporthegrisea* ,which evolve the effector AVR-Pita[20].Interaction of the functional portion of AVR-Pita with the LRR like domain of Pi-ta could be detected by Yeast two-hybrid experiments. Another model was examined which was support the direct detection method ,that the observation in *Arabidopsis thaliana* RRS1 protein interacts with the bacterial wilt pathogen protein PopP2 in a 'split-ubiquitin', which was proved by yeast two hybrid experiment[21]. RRS1 is an atypical member of the TIR-NBS-LRR class of resistance proteins because it contains a carboxy-terminal WRKY domain[22]. Notably, the inactive form of RRS1, RRS1-S, can also bind to PopP2 in that assay, suggesting that either the interaction in yeast does not recapitulate the interaction in plants or that steps in addition to ligand binding are necessary for the activation of signaling.

The example of an indirect recognition mechanism in the Arabidopsis thaliana is,arabidopsis proteins RPS5and PBS1 detected the *P. syringae*effector AvrPphB. RPS5 is a plant NBS-LRR,whereas PBS1 is a protein kinase with unknown substrates [23–24]. Both proteins are requiredfor the recognition of AvrPphB in *P. syringae*strains. Direct interaction between RPS5and AvrPphB has not been detected; however, the interactionbetween both AvrPphB and RPS5 with PBS1 and resulting a ternary complex (J. Ade and R.W.I). AvrPphB is a cysteineprotease which cleave PBS1 at a specific site [25, 26]. Therefore, itseems that RPS5 functions to detect pathogen effectors such as AvrPphB by monitoring thestatus of PBS1.

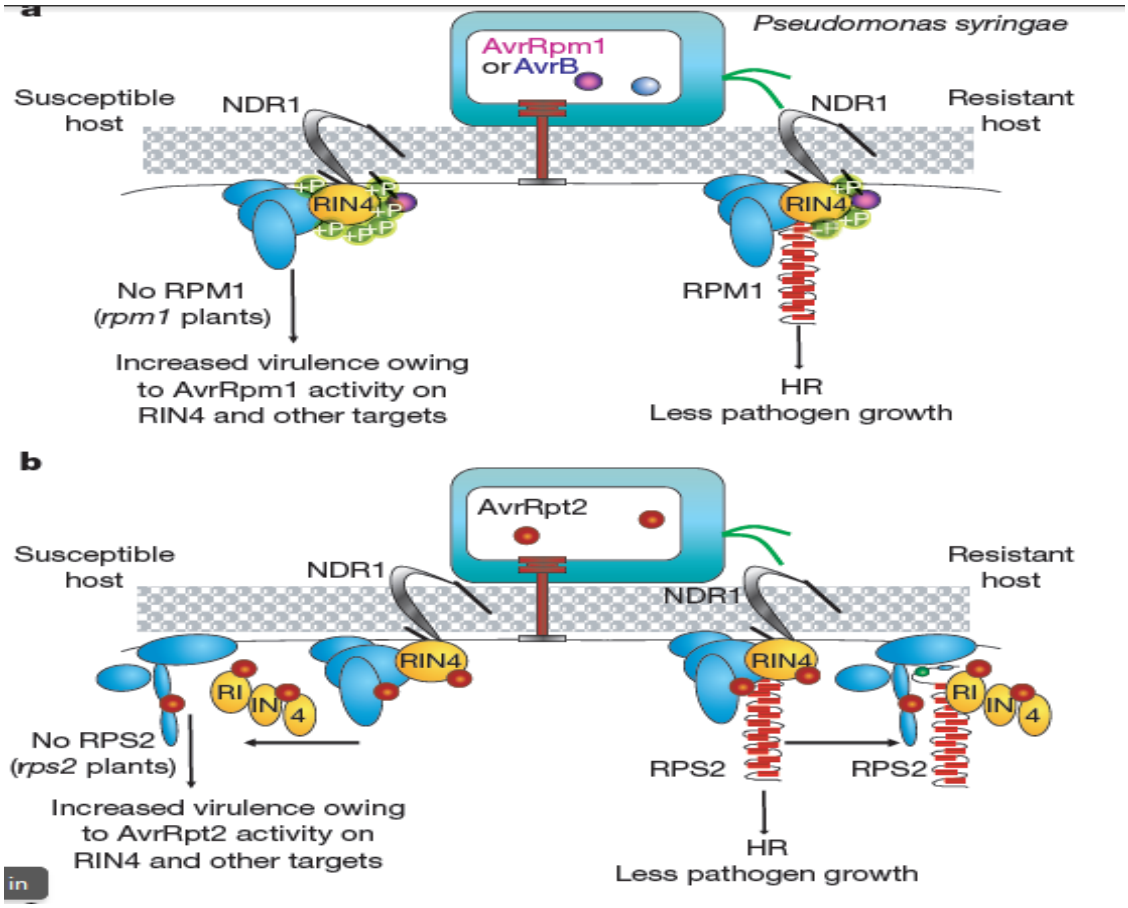
AvrRpm1 and AvrB are two effector protein isolate from the *Pseudomonassyringae* a bacterial pathogen, both are recognized by RPM1 of NBS-LRR protein in *A. Thaliana,*wheras

another type of effector protein from *P. syringae*is AvrRpt2 (cysteine protease type III effector),which is recognized by *A. thaliana* NBS-LRR protein RPS2[27,28].The direct

interaction between effector molecule and *A. thaliana* NBS-LRR protein has not been detected . However, RIN4, another plant protein is linked with AvrB,AvrRpm1 and AvrRpt2[29,30].RIN4 bind to both RPS2

and RPM1 that giving the indirect recognition patterns of AvrRpt2,AvrB andAvrRpm1. The binding patterns provides a functional changes in the RIN4 protein for which RIN4 phosphorylated and proteolytically cleaves by AvrRpt2 [29,30]. Therefore, the

Arabidopsisbasal defence responses is negatively regulated by RIN4 protein.



(FIGURE:6 Example of nonhost resistance)

The location of LRR domains in NBS-LRR protein at the site of carboxy terminal.Crystallization of non-plant protein LRR domains have been occurred, as a result a barrel like structure aligned with parallel β-sheet lining is formed, which is situated at the

inner concave surface and the rest of space is occupied by α-helical structure.LRR domain involved in detection pathogen effector molecule and help to support several evidence in

hypothesis. However, it is thought that protein-protein interaction in animal system is mediated by LRR domain. Pathogen effector targeted a protein in plant which is present with NBS-LRR protein. The amino-terminal domain of the NBSLRR protein mediated the interaction ofpathogen target–NBS-LRR. As a result ,a tightly folded complex structure is formed which is consist of amino terminal domain, NBS,LRR and effector target part. Adenine nucleotide is bound with NBS domain, that confirm the conformation of NBS domain. In particularly the interaction shows negative regulatory function.

Effector induces the conformational changes in host protein and help to exchange of ADP for ATP. That brings the change in nucleotide bounded with NBS domain, which in turn again changes the NBS-LRR domain structural arrangement. Activation in NBS-LRR protein represented the structural changes and binding status of nucleotide. These alteration inducing a new binding sites for downstreamsignaling molecules and signalling pathways activation was resulted.

OBJECTIVES:

- **To screen wild type *Arabidopsis* (nonhost) against infection with *M. oryzae.***
- **To study the infection microscopically: visualize the dead cell and callose deposition due to pathogen entry.**
- **To study the defence pathway genes by RT-PCR**

MATERIALS & METHODS:

SAMPLE PREPARATION

Plant material:

Arabidopsis seeds were collected from "Nottingham Arabidopsis Stock center" (NASC) and stored at 4°C. The *Arabidopsis* accession code was Col-0. I used the wild type plant(col-0,ler-0, npr, pen2gfp). Then, seed samples were soaked in distilled water in a 1.5 mL eppendorf tube overnight (that is needed for good germination and breaking the dormancy).

Soil preparation:

Agro peat soil were mixed with vermiculite in the ratio 1:5,mixed evenly. Then pots were filled with the mixture of soil.

Fertilizer preparation:

TABLE-2

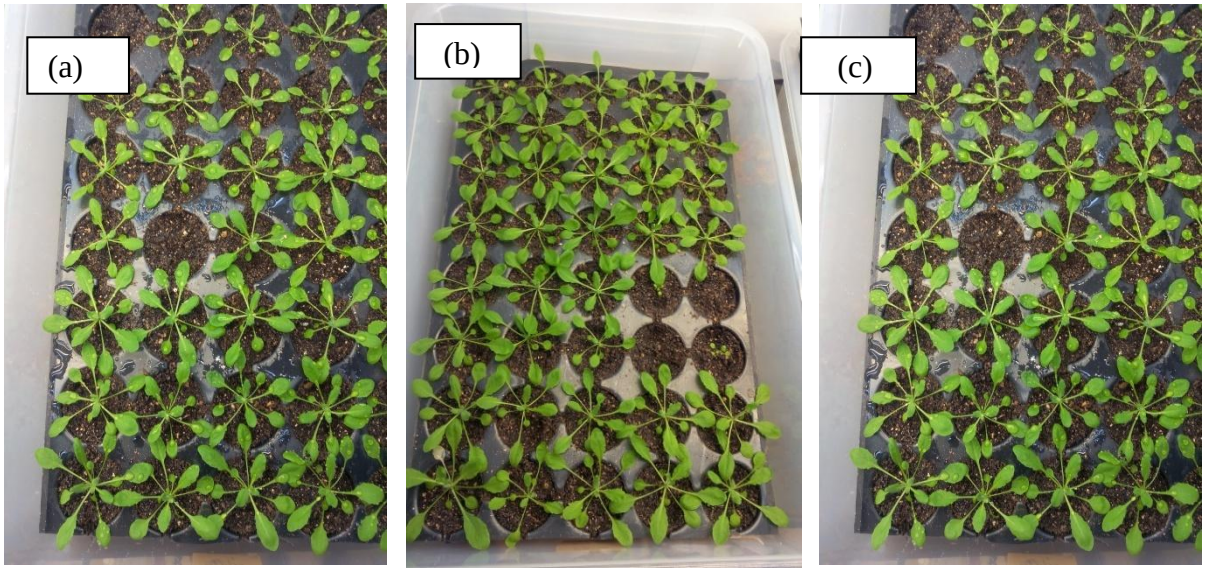
Name of chemical	Amount
Ammonium nitrate	6.516g in 1000ml
Potassium chloride	7.6249g in 1000ml
Disodium hydrogen phosphate	1.824g in 1000 ml

100mL was taken from each stock solution and added water to maintained volume 600mL and pouring each tray.

Plant growth

Seeds were then sowed on mixture of soil then covered it with plastic film so that humidity could be maintained. Light and temperature were maintained. Light should be maintained and

temperature of plant growth chamber was maintained at 21°C. After 3 days, uncovered the tray and seedlings formation occurred. For the better growth of plant water and fertilizer were given to the plant alternately. After 11 days plantlet were transplanted individually in the pot. For maintaining the humidity the tray was covered for three days.



(FIGURE: 7- (a) Col-0 (b) Ler-0 (c) npr)

FUNGAL MATERIAL

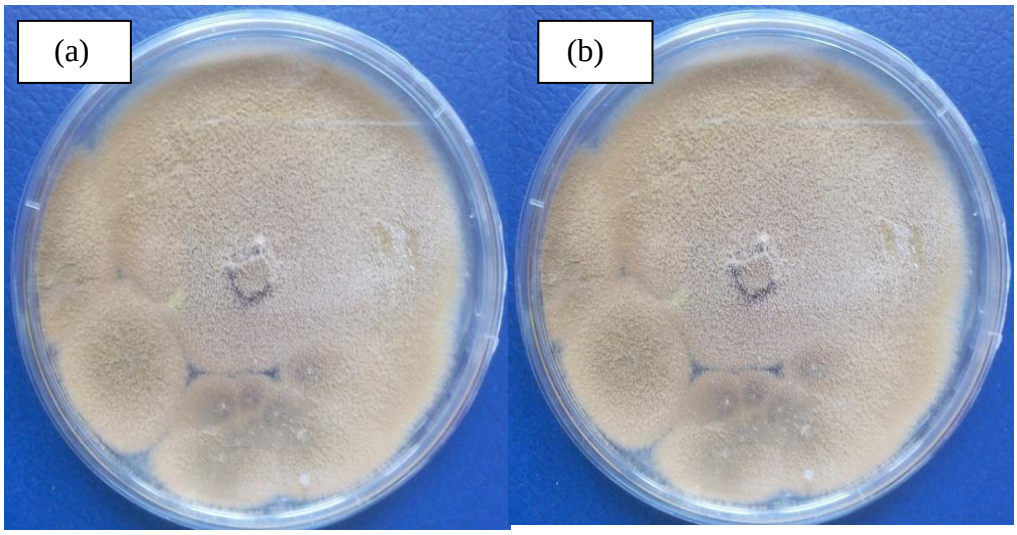
Fungus *Magnaporthe oryzae* was collected from National Center for Plant Genomic Research (NCPGR) of strain Himalayan isolate. *Magnaporthe oryzae* isolate was incubated on oatmeal agar media and potato dextrose agar (PDA) media (with Agar @1.5%) in petridish at 25°C. Then, the inoculum was prepared by washing the petriplates having the mycelia of 7d old growth by distilled water. In order to inoculate *M. oryzae*, spores were diluted 10 µl droplets (10^5 spores/ml). In the culture plates water was added, shake the plate, then transfer the spores in the falcon tube.

Oat meal agar media: (for 100ml)

For 100ml media, 6.00gm of oat meal powder (HIMEDIA) and 1.25 gm of agar (HIMEDIA) was dissolved in 100mL of distilled water and pH of 7.2±0.2 was maintained. Then autoclaved at 121°C at 15 lbs pressure for 15-20 minutes for sterilization. Then 100 µl of streptomycin was added in it before pouring in petridis.

Potato dextrose agar media :(for 100mL)

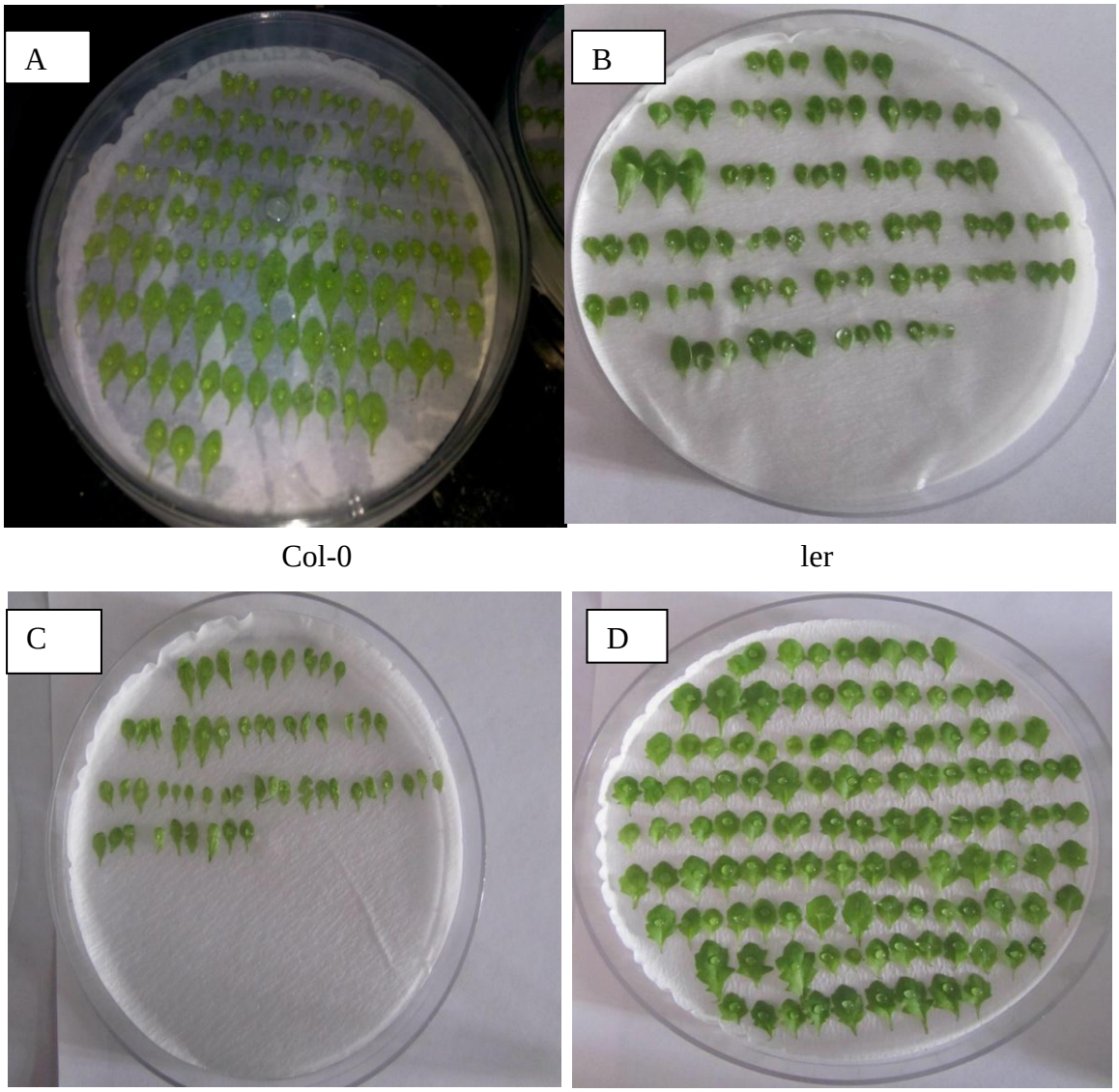
In 100ml PDA media, 1.3 gm of potato dextrose agar (HIMEDIA) and 1 gm of agar (HIMEDIA) was mixed in 100 mL of distilled water. Then autoclaved at 121°C at 15 lbs pressure for 15-20 minutes for sterilization. Then 100 µl of streptomycin was added in it before pouring in petridish. Then about 25µl of media was poured in each petridis.



(FIGURE: 8- (a) patato dextrose agar media (b) oat meal agar media)

LEAF INFECTION

For fungal infection, autoclavable petriplate (150mm* 25mm, HIMEDIA) was taken, Whatman paper was placed on the petriplate. Plant leave of 3-5 weeks old plant was cut and arranged in a triplet. In the first triplet on the moistened paper, water was placed on the surface of the leaf. Then, 10µL of spores was placed on all other triplet leaves. The inoculated leaf sample was then kept at in 25°C and sealed it with parafilm so that humidity could be maintained. Then, after 1 day infection, phenotype was observed.



(FIGURE: 9- (a) col-0 (b)ler (c)pnr (d) pen2-gfp)

STAINING

For analyze the infection we take 1 day infected leaves for staining. Trypan blue stained the dead cell and aniline blue stained the callose deposition.

trypan blue :

- Leaves are taken in 26 well plates and fixed it.
- Fixed sample were rehydrated through decreasing ethanol (100m80,70 and 50% ethanol)
- Samples then were stained in 0.05% trypan blue in distilled water overnight.
- De- staining was done in distilled water in next day.

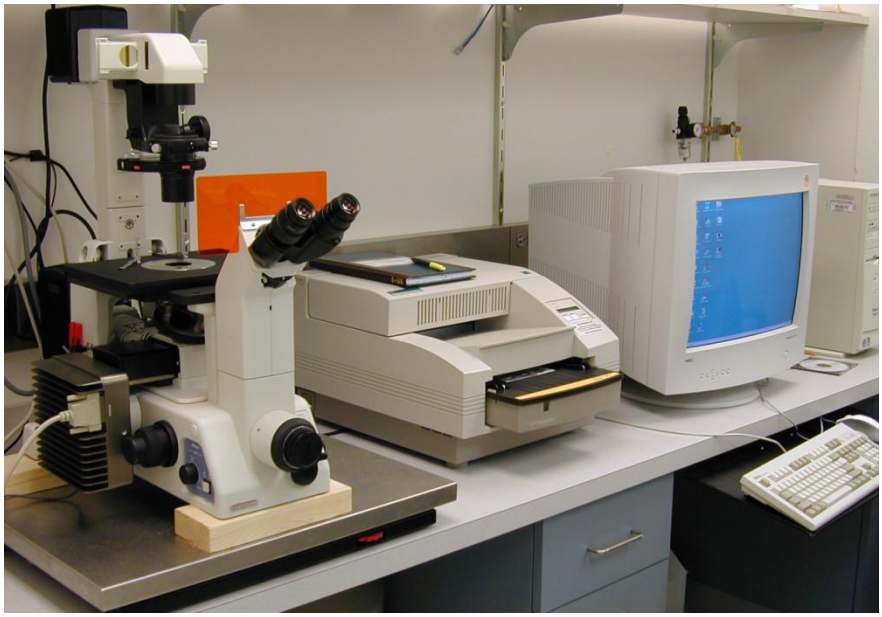
- The leaves then were mounted in 30% glycerol on glass slides.

For visualization the stained cell, slides are observed under fluorescence and taken the images.

Fluorescence Microscopy

A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of reflection and absorption to study properties of organic or inorganic substances. The "fluorescence microscope" refers to microscope that uses fluorescence to generate an image, whether it is a simpler set up like an epifluorescence microscope, which uses optical sectioning to get better resolution of the fluorescent image.

The specimen is illuminated with light of a specific wavelength which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (figure). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images. These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).



(FIGURE: 10-Fluroscent microscope)

Trypan-aniline blue combination

- Leaf samples were re hydrated through decreasing ethanol (100, 80,70, 50%).
- Samples were soaked in 0.05% trypan blue for overnight and then soaked in 0.05%aniline blue in 150mM KH_2PO_4, pH9.5 for 3-4 hr.
- The leaves then were distained in 150mM KH_2PO_4 and 2 to 3 times for 15 minutes and mounted on glass slides.

For visualization the stained cell, slides are observed under confocal microscope and taken the images.

Confocal Microscopy

Confocal microscopy is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

A laser is used to provide the excitation light (in order to get very high intensities). The laser light (blue) reflects off a dichroic mirror. From there, the laser hits two mirrors which are mounted on motors; these mirrors scan the laser across the sample. Dye in the sample fluoresces, and the emitted light (green) gets descanned by the same mirrors that are used to

scan the excitation light (blue) from the laser. The emitted light passes through the dichroic and is focused onto the pinhole. The light that passes through the pinhole is measured by a detector, ie., a photomultiplier tube.So, there never is a complete image of the sample -- at any given instant, only one point of the sample is observed. The detector is attached to a computer which builds up the image.

(FIGURE: 11-Confocal microscope)

DNA ISOLATION BY CTAB METHOD

preparation of stock solution for DNA isolation

2X CTAB buffer (for 10 mL)

NaCl–2.8mL from 5M NaCl stock

Tris HCl -1mL from 1M Tris stock

EDTA – 400µl from 0.5M EDTA stock

CTAB-0.2g

TE buffer(for 10ml)

10mM Tris-100µl from 1M Tris stock

1mM EDTA- 20µl from 0.5M EDTA stock

For DNA isolation, 1 day infected plant was taken.

Procedure for DNA isolation,

- About 100mg tissue was taken and mixed with CTAB buffer and grinding was done in mortal pastel.
- Incubation was done at 65°C about 30 minutes and cool at room temperature.
- About 700µl chloroform was added and vertexed at gently.
- Spinning was done at 12000g about 10 minutes in room temperature.
- Aqueous phase was taken.
- Isopropanol was added about 700µl and mixed well.
- Kept at room temperature and spinning at 12000g about 10 minute in room temperature.
- Supernatant was discarded.
- Ethanol (75%) was added about 500µl to pellet and spinning at 12000g for 3 minutes.
- Supernatant was discarded and pellet was air dry at room temperature about 2 minutes.
- About 20µl TE buffer was added to dried the pellet.

For visualization the DNA bands, DNA runs onto the agarose gel electrophoresis.

AGAROSE GEL ELECTROPHORESIS

- DNA was checked through agarose gel electrophoresis. For preparing 0.8% gel, about 40 mL of 0.5X TBE buffer (5X TAE: 54g Tris , 21.5g boric acid, 20 ml 0.5M EDTA, pH 8) was mixed with 0.32gm of agarose and then the flask was covered with a film paper to avoid loss of liquid due to evaporation and boiled in microwave.
- Then it was kept for cooling and after that 1µl EtBr was added to it.
- It was poured onto the gel casting tray (BIO-RAD) and waited for a while until it was solidified.
- Then the DNA was loaded onto the well and it was run in TBE buffer with 8V/cm.
- After the gel was run (identified by the tracking dye, blue dye migrated upto 2/3rd of the gel length) then it check in the gel doc (BIO-RAD). DNA bands were documented in geldoc (BIO-RAD).

RNA ISOLATION

For RNA isolation,1 day leaves sample was used.

- About 300mg plant tissue was taken and grind in liquid nitrogen.
  Powdered tissue was mixed with about 1ml buffer A: Phenol which is highly heated at 80°C.
- Vertex was done about 5 minutes.
- About 500 µl chloroform was added and vertex about 5 minutes.
- Spinning was done at 12000g about 10 minutes.
- Aqueous phase was transferred to a fresh tube.
- About 500µl chloroform was added and spinned at 12000g about 5 minutes.
- Aqueous phase was transferred into a fresh tube.
- About 500µl 4M LiCl was added to the solution and vertex was done about 3 minutes.
- The tube was incubating overnight at -20°C.
- After overnight incubation spinning was done at 14000g about 20 minute in 4°C.
  Supernatant was discarded and pellet was resuspened in 300µl TE Buffer.
  Ethanol (100%) and NaOAc (3M) were added about 750µl and 30µl respectively.
- Incubation was done about 45 minutes at -20°C.
- After incubation spinning was done at 14000g about 20 minutes in 4°C.
  Supernatant was discarded and about 500µl ethanol (70%) was added to pellet.
  Spinning was done about 14000g about 10 minute in 4°C and supernatant was discarded.
- Pellet was resuspended with 20µl DEPC water and store at -80°C for future use.

In order to visualizethe RNA band, agarose gel electrophoresis was done.

DEPC treated water (1000ml)

About 1000ml of distilled water was taken and 1ml of DEPC (diethylpyrocarbonate) was added in it. Stirring was done overnight by magnetic stirrer. Autoclaved it then repeated the above step once more and then it is ready for use.

PREPARATION OF STOCK SOLUTION FOR RNA ISOLATION

Buffer A: Phenol(10ml)

Requirement:

8M LiCl :- 125µl

0.5M EDTA :- 200µl

20% SDS:- 500 µl

1M Tris pH9:- 1000 µl

DEPC treated water:- 8.175ml

Phenol:- 10ml

8M LiCl (125µl), 0.5M EDTA(200µl), 20% SDS(500 µl) and 1M Tris pH9(1000 µl) was added one by one in a falcon tube then maintained the volume by adding DEPC treated water. After that equal volume of phenol was added in it. Before using it should be kept in the water bathat 80°C.

Phenol:- 10ml

PREPARATION OF STOCK SOLUTION FOR DNA ISOLATION

2X CTAB BUFFER(for 10 ml)

NaCl–2.8ml from 5M NaCl stock

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EDTA – 400µl from 0.5M EDTA stock

CTAB-0.2g

TE BUFFER(for 10ml)

10mM Tris-100µl from 1M Tris stock

1mM EDTA- 20µl from 0.5M EDTA stock

FERTILIZER

Name of chemical	Amount
Ammonium nitrate	6.516g in 1000ml
Potassium chloride	7.6249g in 1000ml
Disodium hydrogen phosphate	1.824g in 1000 ml

100ml was taken from each stock solution and added water to maintained volume 600ml and pouring each tray.

AGAROSE GEL ELECTROPHORESIS

- DNA was checked through agarose gel electrophoresis. For preparing 0.8% gel, about 40 mL of 0.5X TBE buffer (5X TAE: 54g Tris, 21.5g boric acid, 20 ml 0.5M EDTA, pH 8) was mixed with 0.32gm of agarose and then the flask was covered with a film paper to avoid loss of liquid due to evaporation and boiled in microwave.
- Then it was kept for cooling and after that 1µl EtBr was added to it.
- It was poured onto the gel casting tray (BIO-RAD) and waited for a while until it was solidified.
- Then the DNA was loaded onto the well and it was run in TBE buffer with 8V/cm.
- After the gel was run (identified by the tracking dye, blue dye migrated upto 2/3rd of the gel length) then it check in the gel doc (BIO-RAD). DNA bands were documented in geldoc (BIO-RAD).
  discarded.
- Pellet was resuspended with 20µl DEPC water and store at -80°C for future use.

In order to visualizethe RNA band, agarose gel electrophoresis was done.

DNAse treatment

About 0.1 volume of NaOAc and 2.5 volume of DNase TREATMENT:

- About 20µl RNA was taken.
- About 7µl DNase Buffer (10X) and 1µl DNase were added to RNA.
- Incubation was done at 37°C for 30 minutes and added DEPC water to maintained the final volume about 200µl.
- About 200µl phenol :chloroform (1:1) was added and vertexed.
- Spinning was done at 12000g for 10 minutes.
- Upper aqueous phase was transferred to fresh tubes.
- Choloform was added about 200µl and vertexed.

cDNA preparation

First of all, 18µl of template RNA was mixed in 1µl primer

Then, RNA sample was incubated on 70°C for 2 minutes

After that, sample was placed in Ice for 2 minutes

10µL of buffer plus 5µl of dNTP was added in RNA sample

Then 1µl of Reverse Transcriptase was added in it

Then, 15µl of DEPC treated water was added in the RNA sample and mixed it.

Then the sample was run on PCR.

Table: cDNA Protocol

Reaction vol 50µl	Time
25°C	10 min
37°C	1: 30:20
75°C	15 min
10°C	∞

After this we got the cDNA, then we run the sample in normal PCR

PCR (Vol- 10µl)	Time
94°C	3 min
94°C	30 sec
55°C	20 sec 34 cycle
72°C	45 sec
72°C	10 min
4°C	∞

After completing the PCR, cast 2% agarose gel in 1X TAE buffer, load the sample and run. The gel should run upto two-third. Then observed the band in UV trans-illuminator.

PRIMER:

Oligo name	Len	MW	Tm	Mg/OD	OD	µg	nmol	2ndry	GC%	Ml for 100 µm	Seq
UBQ10	22	67	63	31.8	18	599	89.	Very	54.	891	GGCCTTGTATAATCCC

F		25	.7		.8	.5	1	weak	4		TGATGA
UBQ10R	22	6868	60.5	27.5	17.3	476.5	69.3	none	36.3	693	AAAGAGATAACAGGACGGAAA
EF-1aF	22	6643	67.9	35.4	9.4	333.5	50.2	moderate	50	502	TGAGCACGCTCTTCTTGCTTTC
EF-1aR	22	6772	67.8	32.6	14.8	482.8	71.2	weak	50	712	GGTGGTGGCATCCATCTTGTTA
FRK1F	19	5871	59.9	29.8	8.7	259.5	44.2	none	52.6	442	GCCAACGGAGACATTAGAG
FRK1R	20	6006	59.6	32.0	12.2	391.4	65.1	none	50	651	CCATAACGACCTGACTCATC
NHL10F	20	5997	63.6	32.8	21.8	716.7	119.5	none	50	1195	TTCCTGTCCGTAACCCAAAC
NHL10R	20	6118	63.7	32.1	17.5	562.3	91.9	weak	60	919	CCCTCGTAGTAGGCATGAGC
CYP81F2F	22	6835	63.0	28.7	14.2	407.9	59.6	none	40.9	596	AAATGGAGAGAGCAACACAATG
CYP81F2R	20	6012	63.4	32.3	14.7	475.1	79.0	Very weak	45	790	ATCGCCCATTCCAATGTTAC
PR1F	22	6825	67.9	31.2	14.2	443.1	64.9	none	54.5	649	AAAACTTAGCCTGGGGTAGCGG
PR1R	24	7199	66.2	33.6	15.3	514.4	71.4	none	45.8	714	CCACCATTGTTACACCTCACTTTG
PDF1.2aF	22	6858	66.7	29.8	11.7	348.8	50.8	Very weak	50	508	AGAAGTTGTGCGAGAAGCCAAG
PDF1.2aR	23	7160	66.8	31.5	13.3	419.8	58.6	Very weak	52.1	586	GTGTGCTGGGAAGACATAGTTGC

RESULT:

Phenotypic study after infection of plant

Table no:3(col o)

RRR	RRR	RRR	RRR
RRR	SSS	RRR	RRR
RRR	RRS	RRS	RRS
RSS	RRR	RRS	RRR
RRS	RRR	RRS	RSS
RRR	RRR	RRR	RRR
RRS	RRR	RRR	RRR
RRR	SSS	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRS	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR

RRR			

LER0

RRR	RRR	RRR	RRS
RRR	RRR	RRS	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRS
RSS	RRR	RRR	RRR
RRR		RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRS	

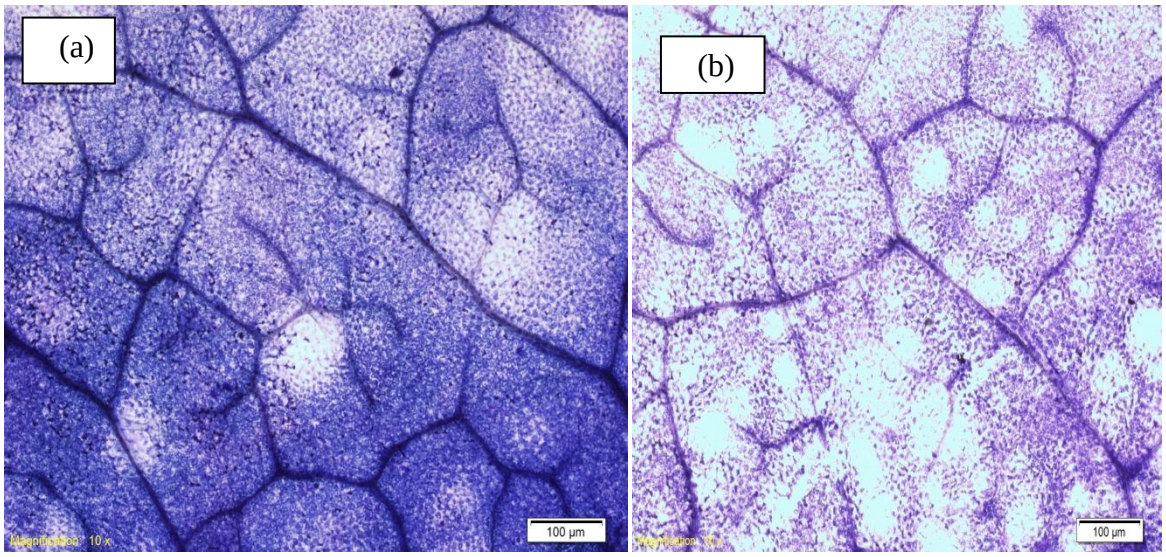
PEN2 GFP

RRR	RRR	RRR	RRR
_RR	RRR	RRR	RRR
RRR	RRR	RRR	R_ _
RR_	RRR	RRR	RRR
RRR	RRS	RRR	RRR
RRR	RRR	R_ _	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RR_
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR			

NPR

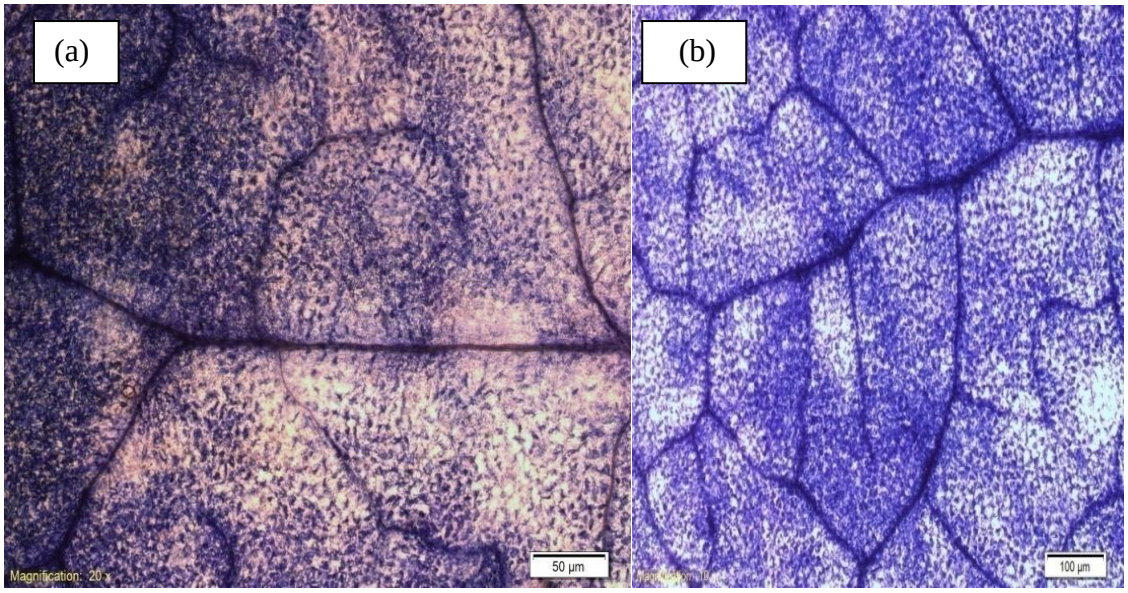
RRR	RRR	RRR	RRR
RRS	RR	RRS	RRS
RR_	RSS	RRS	RRR
RR_	RR_	RRR	

Fluroscent microscopic images of the leafe sample:



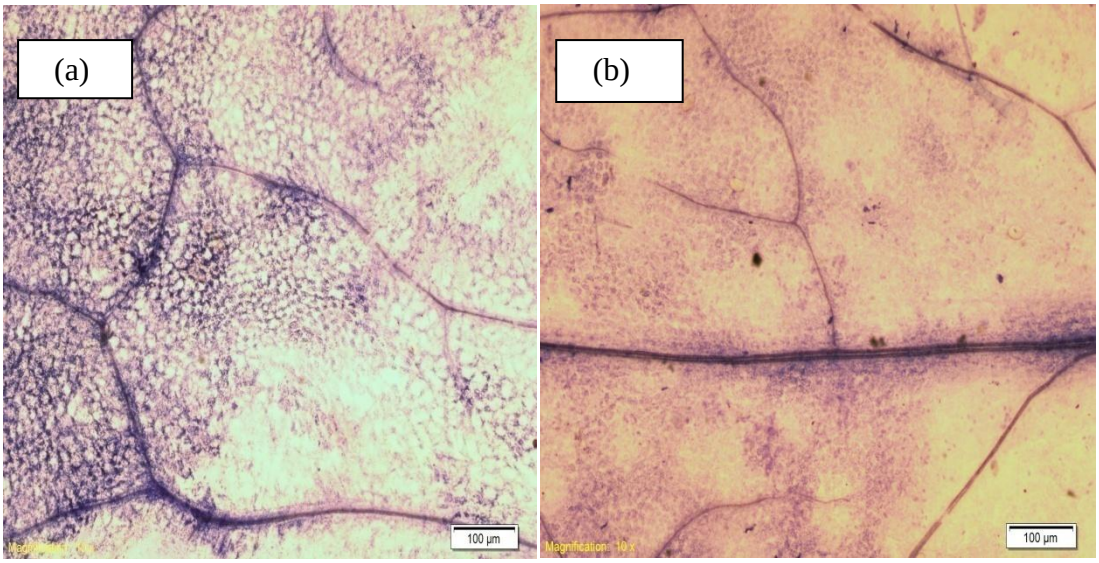
Col-o 1d col-o(water)

(FIGURE: 12-(A)col-o one day infection, (b)col-o water)



Ler 1d Ler(water)

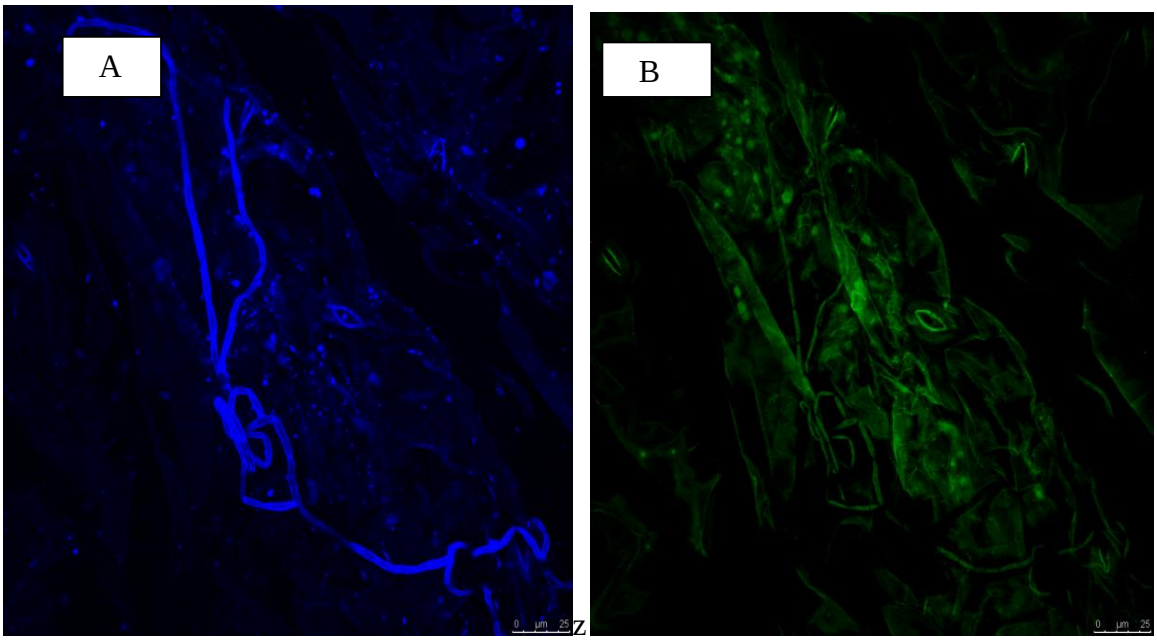
(Figure:13-(a)= ler one day infection,(b)ler(water)



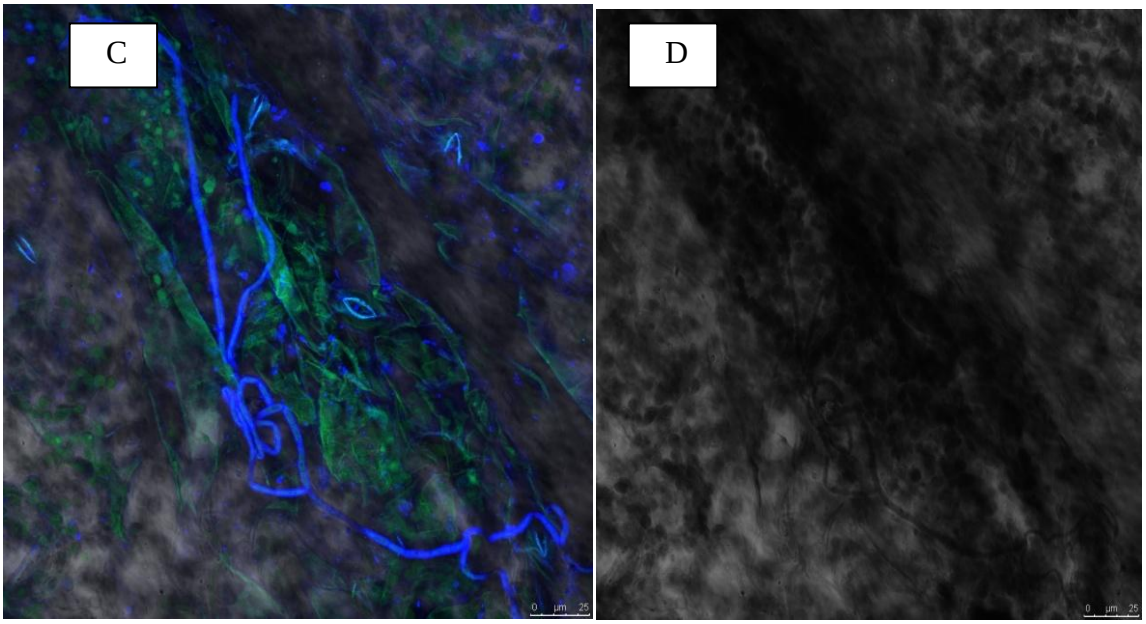
NPR 1d NPR(water)

(Figure: 14-(a) NPR In one day infection,(b)= NPR (water))

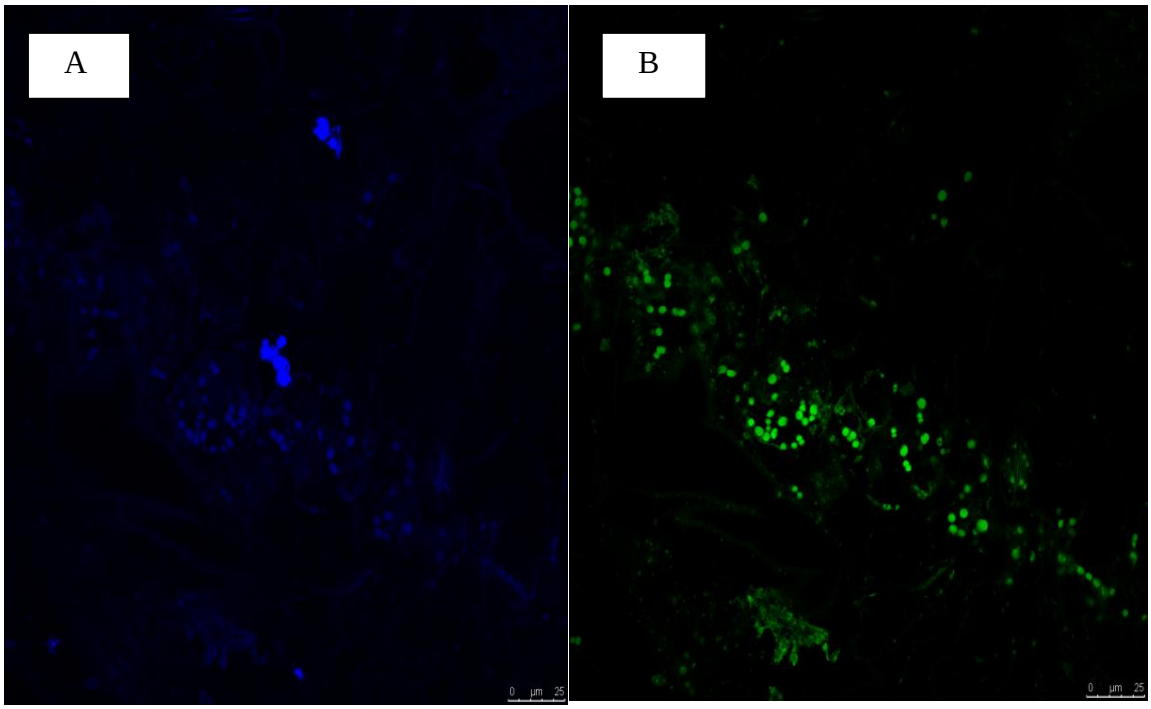
Confocal microscopy images of the leaf sample:

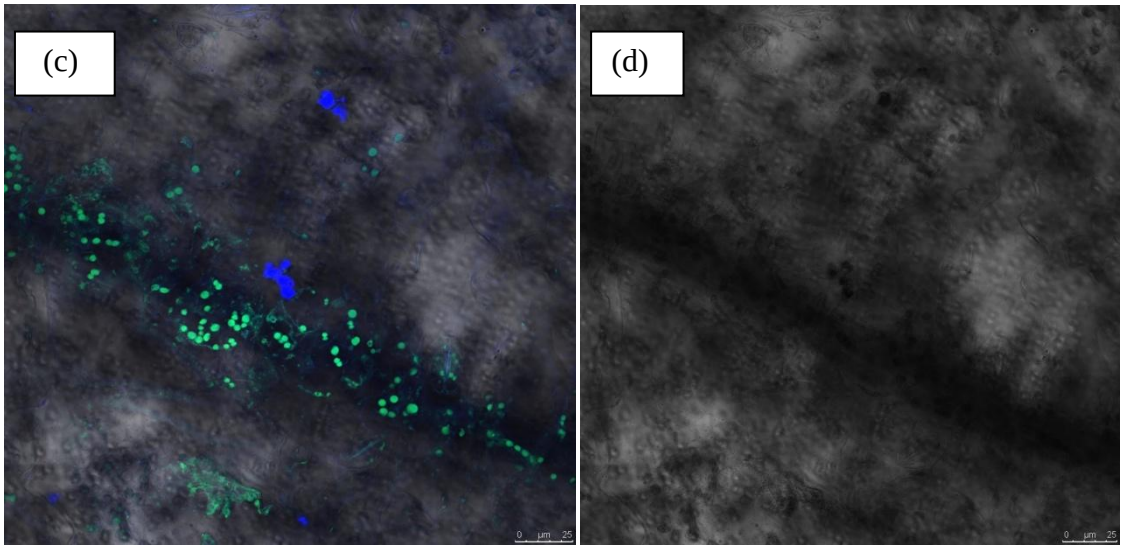


(FIGURE:15-(a) col-0 infection with anelene stain (b) col-0 infection with trypan blue stain)



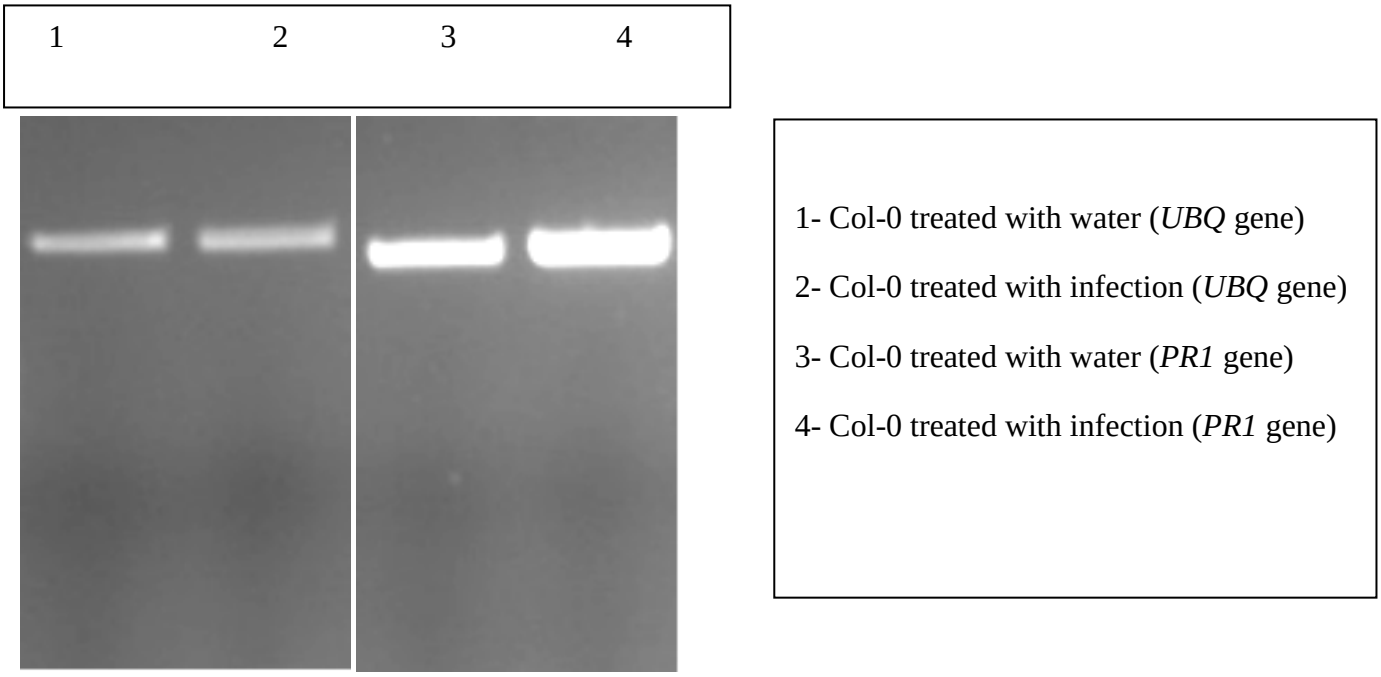
(FIGIRE: 16 -col-0(c)merged stain image, (d)DIC stain image)





(FIGURE:17-Images of Ler-0 (a)anelene blue stain ,(b)trypan blue stain, (c)merged image stain ,(d)DIC stain image)

To check the expression pattern of the defence pathway genes in (col-0 and ler-0) by RT-PCR method.



(FIGURE:18-expression pattern of the PR gene)

- In this picture , first & second band shows that UBQ gene expression in both water treated & in infection in same base pair. But in case of third & fourth band the PR gene is more expresses in infection sample.
- More expression of PR gene,more resistant to the pathogen.

- So here we found that the PR gene is resistant the pathogen in infection side of the plant.

- **CONCLUSION:**

- So i concluded from the overall experiment that the PR gene expression is more in wild type plant,so it shows more resistant to the pathogen.
- Wild type plants (Ler-0) are resistant to the pathogen and can be taken as a true control as a nonhost against *M. Oryzae* for further studies.
- The wild type plants are easily not infected by the pathogen.

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